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FOREWORD

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David A. Hamer
PI - Signature

6/28/98
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Introduction

The treatment of patients with advanced breast cancer using chemotherapeutic agents is often limited due to the inherently toxic nature of these drugs to rapidly dividing tissues (1). Tumor specific conversion of an inactive prodrug to a chemotherapeutic agent may overcome this limitation (2). Therefore, we proposed to develop and test a novel enzyme/prodrug strategy for cancer gene therapy. We chose Carboxypeptidase A (CPA) as the therapeutic gene based on its ability to enzymatically convert the prodrug methotrexate- α -phenylalanine (MTX-Phe) to the toxic agent methotrexate (MTX) (3). By targeting expression of CPA to tumor cells it is expected that systemically administered MTX-Phe will be converted to high intra-tumoral concentrations of MTX. This system is expected to lead to heightened tumor cell kill without the associated systemic toxicities. CPA is normally synthesized as a zymogen that requires proteolytic removal of its pro-peptide by trypsin in order to gain catalytic activity (4). The first goal of the proposal was to develop and characterize *in vitro* mutant forms of CPA which would be activated by endogenous intracellular proteases even in absence of trypsin.

In the first year of this award we have developed an improved form of CPA, herein termed CPA_{ST3}, which is endogenously activated even in absence of trypsinization or co-expression of any other activating enzyme. In addition, we have gone on to purify and enzymatically characterize this mutant form of CPA and have demonstrated that it has an identical kinetic profile as the native trypsin activated enzyme. To alleviate any difficulties that might arise from a soluble enzyme which may be released from the tumor and lead to systemic production of MTX from MTX-Phe, we have developed and characterized a cell-surface associated form of CPA which should lead to CPA catalytic activity which is constrained to the tumor. Finally, we are in the process of characterizing the ability of both the soluble and cell-surface forms of CPA to sensitize tumor cells to MTX-Phe *in vitro*.

Body

Material & Methods

Transient Expression: The expression plasmids for wild-type CPA, the two mutants (CPA₉₅ or CPA_{ST3}), PACE, or the dominant negative PACE.SA were transiently expressed in 293T cells or COS-1 cells by calcium phosphate precipitation and DEAE-Dextran respectively. Transfected cells were switched to serum free medium (OPTIMEM, Gibco) 48 hours after transfection and conditioned media were then collected for another 24 hours prior to western blotting, enzymatic assay, or purification.

Enzymatic Assays: CPA activity was measured using a spectrophotometric assay for cleavage of a synthetic substrate, N-(3-[2-Furyl]Acryoyl)-Phe-Phe (FAPP, Sigma) which undergoes a decrease in absorbance at 330 nm after cleavage by CPA.

CPA Purification: CPA was purified from conditioned media using CPA potato inhibitor affinity chromatography or an α -CPA immunoaffinity column. For CPA potato inhibitor chromatography conditioned media were dialyzed against binder buffer (250 mM NaCl, 20 mM Tris, pH 8.0), loaded onto the column, washed with binding buffer, and eluted (1 M NaCl, 0.1 M NaHCO₃, pH 11.4). An α -CPA affinity column was constructed according to the manufacturers instructions (Affi-Gel Hz, Bio Rad). Conditioned media were diluted 1:1 with PBS, loaded onto the column, washed (500 mM NaCl in PBS), and eluted (500 mM NaCl, 20 mM Glycine HCl, pH 2.0).

Retroviral Production: The cDNA for CPA_{ST3} was sub-cloned into Lzrs.pBMN and retrovirus produced by transfecting the ϕ nX-ampho packaging cell line (both courtesy of Gary Nolan, Stanford). SCCVII cells were infected using retroviral supernatants from the packing cell line supplemented with polybrene.

Cytotoxicity Assays: SCCVII cells were seeded in 96 well plates on day -1, on day 0 the cells were infected with retroviral supernatant, and MTX or prodrugs were added on day 1. Cells were left cultured with drugs until control wells were confluent (48-96 hrs) at which time the cells were fixed in 10% TCA, washed extensively with H₂O, stained with sulforhodamine-B, and destained in 1% acetic acid. Fixed dye was then solubilized in 10 mM Tris, and the plates scanned on a 96-well microtiter plate reader (Molecular Devices). "% Growth Inhibition" was calculated against untreated controls; each condition represented in 8-fold replicate..

Results & Discussion

Specific aim 1: Generate stable cell lines that express endogenously activated forms of CPA and evaluate the catalytic activity and substrate specificity of the CPA produced from these lines.

The goal of this aim was to construct CPA molecules that are activated by proteases resident within the golgi compartment of mammalian cells. This would result in the production of mature CPA and therefore CPA activity from transduced cells. The CPA constructs derived in the original grant submission (CPA₉₅) required that the processing enzyme PACE be co-expressed to result in the production of active CPA. Since this would require the transduction of two genes into cells which would not be optimal we began constructing additional mutants of CPA. Indeed, we have accomplished this goal for the newest mutant, CPA_{ST3}, which incorporates a 10 amino acid fragment from the activation segment of the matrix metalloprotease stromolysin 3 (ST3), is fully activated when expressed *in vitro* even without exogenously expressed PACE (5). (see figure 1)

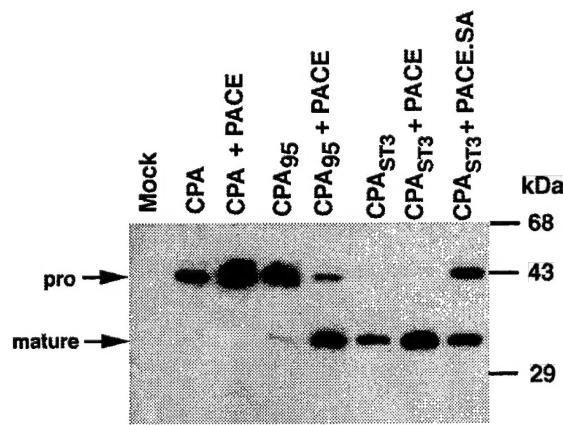


Figure 1: Expression of mature CPA in mammalian cells. Three forms of CPA were analyzed for their ability to become secreted from mammalian cells as mature enzymes in the absence or presence of PACE. Wild type rat CPA (CPA) in the presence(lane 3) and absence of PACE co-expression(lane 2) is not converted to mature CPA. In contrast CPA₉₅ is cleaved to mature CPA only in the presence of PACE expression (lanes 4 and 5). The most recent mutant we have constructed, CPA_{ST3} is secreted as mature CPA from cells in the absence (lane 6) or presence of PACE co-expression (lane 7). To demonstrate that CPA_{ST3} is cleaved by PACE, we co-expressed a dominant negative mutant of PACE which resulted in partial inhibition of CPA_{ST3} processing (lane 8)

We have also begun preliminary biochemical studies to demonstrate that the tissue culture cell derived enzyme is identical to the trypsin activated pancreatic enzyme (see Table I).

Table I. Kinetic analysis of mutant and wild-type CPA

ENZYME	K _m (1/M)	K _{cat} (1/min)
CPA Trypsinized	7.8x10 ⁻⁵ ± 9.2x10 ⁻⁶	13,000 ± 725
CPA ₉₅ Trypsinized	7.6x10 ⁻⁵ ± 7.1x10 ⁻⁶	12,500 ± 550
CPA ₉₅ + PACE	8.5x10 ⁻⁵ ± 8.4x10 ⁻⁶	12,750 ± 600
CPA _{ST3}	8.2x10 ⁻⁵ ± 9.6x10 ⁻⁶	12,250 ± 1135

Hydrolysis of FAPP by 2.0 x 10⁻¹⁰ moles trypsin activated CPA, trypsin activated CPA₉₅, PACE activated CPA₉₅, or CPA_{ST3} was monitored at 330 nm in assay buffer (pH 7.5, 50 mM Tris-HCl, 0.45 M NaCl, 25°C). Kinetic constants were calculated by non-linear regression and are given as the mean of three trials ± the standard error of the mean. Specific activity was determined at 8.0 x 10⁻⁵ M FAPP using bovine CPA as a standard.

In addition, since mutations in the propeptide of CPA have been shown to greatly alter the catalytic activity of the protease we wanted to assure that the substrate specificity was not altered from that reported in the literature. Therefore, cytotoxicity assays were undertaken using a number of different MTX-α-peptide

prodrugs following retroviral transduction of cells in culture. These assays demonstrate that there is no significant alteration in the substrate specificity of CPA_{ST3} as compared to wild-type CPA and that MTX-Phe is the best substrate in this system with a 75-fold increase in cytotoxicity in the presence of CPA. (see Table II) (6,7).

Table II. Cytotoxicity of MTX- α -peptide prodrugs following retroviral transduction

<u>IC₅₀ (nM)</u>	<u>LacZ</u>	<u>CPA_{ST3}</u>	<u>Factor</u>
MTX	25	N/A	N/A
MTX-Trp	4000	4000	1
MTX-Gln	3000	2000	1.5
MTX-Met	6000	2000	3
MTX-Tyr	5000	450	11
MTX-Phe	15,000	200	75

SCCVII cells, a mouse squamous cell carcinoma, were infected with a retrovirus driving expression of CPA_{ST3} or a control (LacZ) retrovirus. Infected cells were then exposed to varying concentrations of different α -peptide MTX prodrugs (a gift from M. Page, University Lavel, Quebec, Canada) and the cytotoxicity under these conditions monitored by sulforhodamine-B assay in 96 well tissue culture plates. Data represent the mean of three experiments.

As discussed in the original grant submission we believe that a secreted form of CPA may have the advantage in that it will result in the production of MTX within the tumor milieu thus exposing transduced and non-transduced cells to similar concentrations of the drug. This would result in an enhanced bystander effect. Unfortunately, a disadvantage of this is that CPA could travel outside of the tumor site and result in the production of MTX from MTX-Phe within normal tissue. To overcome this we have constructed a cell surface form of the enzyme based upon the phosphatidylinositol linkage in decay accelerating factor (see figure 2) (8).

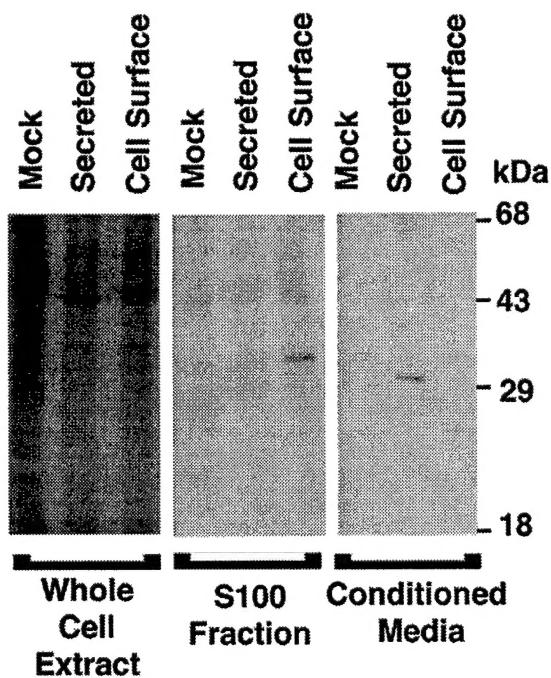


Figure 2: Comparison of cell surface and secreted CPA. Mock transfected cells, cells transfected with the secreted form of CPA (**secreted**) and cells transfected with the cell surface form of CPA (**cell surface**) were isolated as was the conditioned media. To confirm that the expected proteins were being expressed, cells were analyzed for the presence of CPA (**whole cell extracts**) and as expected the secreted form of CPA was synthesized as a 43 kDa band inside the cell while the cell surface form was synthesized as a 45 kDa band. The cell pellet was dounced and the cell membranes were isolated by a 100,000g spin. The membrane fraction (**S100**) was also analyzed for the presence of CPA and only the cell surface form of CPA was detected in the S100 fraction indicating that it and not the secreted form was localized to the membranes. When the **conditioned media** was analyzed only the secreted form of CPA was detected as expected.

Further characterization of both the soluble and cell-surface forms of CPA are being performed to complete the experiments outlined in specific aim 1. However, preliminary data support the conclusion that all major goals outlined in specific aim 1 and slated for completion during year one of the proposal have been completed.

Specific aim 2: The cytotoxicity of MTX-Phe in the absence or presence of CPA will be evaluated *in vitro*. In addition, the ability of a small number of CPA expressing cells to sensitize other non-expressing cells to MTX-Phe will be explored in order to characterize a “bystander effect.”

To evaluate the ability of retroviral transduction of both soluble and cell-surface forms of CPA to sensitize cells to MTX-Phe cytotoxicity experiments were undertaken (figure 3).

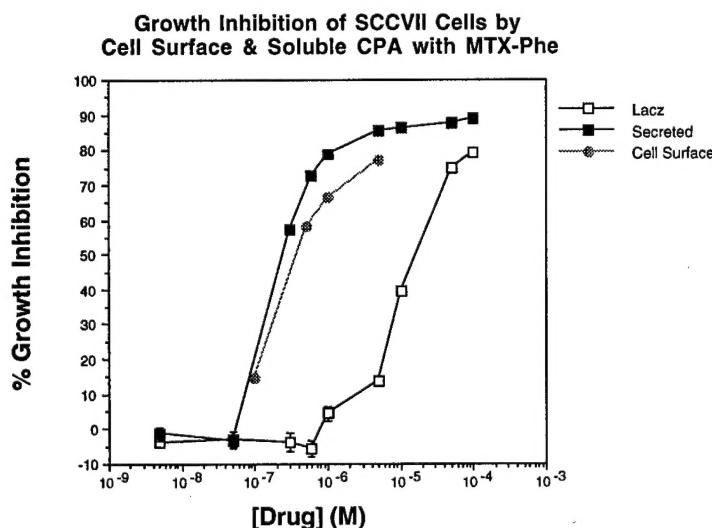


Figure 3: Expression of secreted CPA or cell surface CPA results in sensitization of SCCVII cells to MTX-Phe. To test if expression of the secreted or cell surface forms of CPA resulted in MTX-Phe dependent cytotoxicity we used a growth inhibition assay as described in Materials & Methods.

Since retroviral infection only leads to a partially transduced population of cells this experiment already points to the “bystander effect” present in this system. However, in order to more carefully evaluate the level of “bystander” killing we are currently using stable cell lines to precisely regulate the proportion of transduced and non-transduced cells in similar experiments.

Conclusions:

- 1.) CPA_{ST3}, an endogenously activated form of CPA which does not require trypsinization or co-expression of PACE in order to become catalytically active, has been developed.
- 2.) This mutant has been purified and characterized biochemically demonstrating that it has an identical kinetic profile as the trypsin activated wild-type enzyme.
- 3.) Cytotoxicity assays indicate that the mutant enzyme has a similar substrate specificity as the wild-type enzyme.
- 4.) A cell associated form of CPA_{ST3} has been developed which is also catalytically active.
- 5.) Retroviral constructs have been developed and recombinant retrovirus produced which allow infected cells to express either soluble or cell-surface forms of CPA, and both of these retroviruses effectively sensitize tumor cells *in vitro* to MTX-Phe.

Statement of Work:

The project appears to be progressing well and in accordance with the plan outlined in the original Statement of Work. No alterations in the time frame of the proposal are warranted at this time.

References:

1. Osteen, R.T. et al. 1990 *Cancer Manual*. 8th ed. Amer Cancer Society, Boston, MA.
2. Connors, T.A.. 1995. *Gene Therapy*, 2:702-709.
3. Vitols, K.S. et al. 1995. *Cancer Res*, 55:478-8.
4. Oppezzo, O. et al 1994. *Eur. J.Biochem*, 222:55-63.
5. Pei, D. and Weiss, S.J. 1995. *Nature* 375, 244-247.
6. Phillips, M.A. et al. 1996. *J. Biol . Chem.*, 35:6771-6776.
7. Perron, M.J. et al. 1992. *In J Oncol*, 5:907-913.
8. Caras I.W., Weddell G.N. 1989. *Science* 243, 1196-1198.